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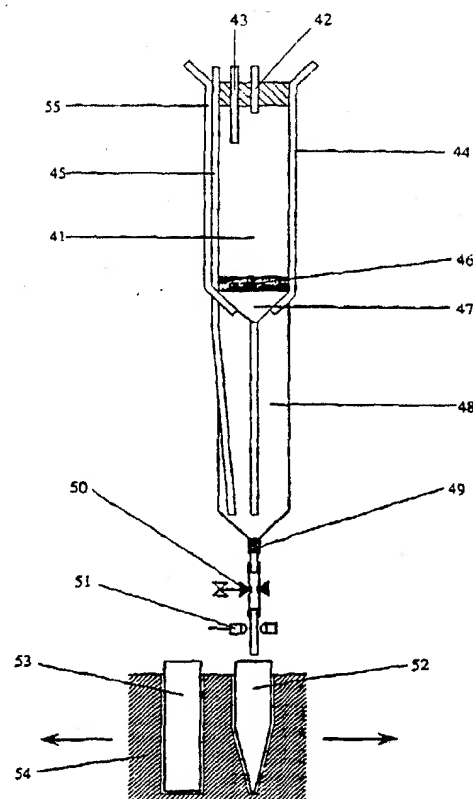
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(21) International Application Number: PCT/GB94/01484 (22) International Filing Date: 8 July 1994 (08.07.94) (30) Priority Data: 9314249.5 9 July 1993 (09.07.93) GB (71) Applicant (for all designated States except US): CAMBRIDGE MOLECULAR TECHNOLOGIES LIMITED [GB/GB]; 13 Station Road, Cambridge CB1 2JB (GB). (72) Inventor; and (75) Inventor/Applicant (for US only): JONES, Christopher, Peter [GB/GB]; 84 Fowlmere Road, Heydon, Herts SG8 8PU (GB). (74) Agents: DANIELS, Jeffrey, Nicholas et al.; Page White & Farrer, 54 Doughty Street, London WC1N 2LS (GB).		(81) Designated States: AM, AT, AU, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, ES, FI, GB, GE, HU, JP, KE, KG, KP, KR, KZ, LK, LT, LU, LV, MD, MG, MN, MW, NL, NO, NZ, PL, PT, RO, RU, SD, SE, SI, SK, TJ, TT, UA, US, UZ, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG), ARIPO patent (KE, MW, SD).  <b>Published</b> With international search report.	

(54) Title: PURIFICATION METHOD AND APPARATUS

(57) Abstract

A method is provided for purifying from cells a target compound such as nucleic acid. The method comprises the following steps: 1) lysing a cell suspension to form a cell lysate containing the target compound; 2) applying the cell lysate to a filter to remove unwanted cells and cell debris; 3) contacting the filtered lysate with a solid phase matrix under conditions to bind the nucleic acid to the matrix; 4) separating the resultant filtered lysate from the matrix; and 5) eluting the target compound from the matrix. Apparatus is also provided. Complex purification procedures such as centrifugation are avoided.



### PURIFICATION METHOD AND APPARATUS

The present invention relates to a method and apparatus for purifying target compounds such as nucleic acids from cells.

Conventional procedures for the purification of protein or nucleic acid, such as DNA, require lysis of the source cells followed by various fractionation steps involving centrifugation. Where DNA manipulation is to be carried out, small scale DNA preparations are required routinely, often in large quantities for the purpose of screening the DNA from the source cells. These processes are time consuming and labour intensive.

It has been proposed to avoid centrifugation in the extraction and purification of DNA by a relatively complicated series of steps. EP 0376080 discloses in general terms the use of precipitants to precipitate DNA from accompanying impurities. Ultrafiltration is then used as a means to isolate the DNA. However, no worked example of the proposed process is described and no indication of its feasibility is indicated. Ultrafiltration forms the basis of other DNA purification methods as described in WO 87/07645 and EP 0517515.

Cation exchange resins have also been proposed as a means for separating relatively uncontaminated nucleic acids from impurities. EP 0281390 discloses the use of polycationic solid supports particularly to separate hybridized from unhybridized nucleic acids. EP 0366438 discloses the separation of nucleic acid from protein by binding the protein to a cation exchange resin.

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from batch of cell suspension to desired product), for example in automated apparatus.

In a further aspect, the present invention provides a continuous method for purifying a target compound from cells. The method comprises the following steps:

- (1) obtaining a cell suspension containing the target compound;
- (2) applying the suspension to a filter to remove unwanted cells and cell debris;
- (3) contacting the filtrate with a solid phase matrix under conditions to bind the target compound to the matrix;
- (4) separating the resultant filtrate from the matrix; and
- (5) eluting the target compound from the matrix.

Preferably, the cell suspension is lysed in step (1) to form a cell lysate containing the target compound.

Preferably, the method further comprises the step of washing the matrix binding the target compound so as to remove contaminants before eluting the target compound from the matrix.

Any cell producing the target compound may be used in the method. For the purpose of this specification, the term "cell" is intended to encompass bacterial cells, cells from higher organisms, phage particles and other cell types or organelles which contain the target compound and may require some form of lysis step to release it. In the case of bacteria, nucleic acid may come from the bacterial nucleus or from cellular inclusions such as plasmids. Indeed, the method is especially useful for the

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The step of lysing the cell suspension typically requires the addition of a further aliquot of lysis buffer. After lysis, it is sometimes preferable to add a neutralization solution to the lysed cell suspension. Typically, the buffers for resuspension and lysis and the neutralization solution are all well-known in this field and can vary according to the cell type and target compound to be purified. For example, where nucleic acid is to be purified the resuspension buffer typically contains a chelating agent to remove metal ions from the medium and may have a pH in the range 7 to 8.5, advantageously in the range 7 to 8. For plasmid purification the lysis buffer is typically alkaline and includes a surfactant such as sodium dodecylsulphate (SDS). The neutralization solution is intended to bring the pH back into a useful range and can flocculate unwanted protein and is typically a highly concentrated salt solution such as potassium acetate at around 2.5 M. For nucleic acid purification from blood cells, the lysis buffer includes Nonidet P-40 as a typical surfactant and the pH is preferably raised to around pH 8.3.

The nucleic acid may be DNA or RNA. Where DNA is to be purified, the resuspension buffer may contain RNase to cleave unwanted RNA. Where RNA is to be purified the resuspension buffer may contain DNase to cleave unwanted DNA. Optionally, for nucleic acid purification the resuspension buffer may also contain proteases, for example proteinase K.

At each stage of adding the resuspension buffer, lysis buffer, and neutralization solution, it is preferable to mix the solutions well together.

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In each case, the conditions of pH and salt concentration will affect both the ability of the target compound and the unwanted contaminants to bind to the matrix. It is therefore advantageous to include the step of washing the matrix prior to elution so as to remove unwanted contaminants which bind less strongly to the matrix than the target compound. A typical wash buffer for nucleic acid is alcoholic Tris-HCl at around pH 7.5 with 200 mM sodium chloride and 5mM EDTA.

The contacting of the filtrate with the solid phase matrix may occur simply by passing the filtrate through a volume of the matrix, perhaps in the form of a column, for example. Alternatively, a suspension of the matrix may be added to the filtrate. Separation of the thus-treated resultant filtrate from the matrix may be effected simply by retaining the matrix and discarding the unwanted liquid phase.

Elution of the target compound from the matrix will depend upon the nature of the interaction between the compound and the matrix. Where ionic interactions predominate, it is usual to elute the nucleic acid in an elution buffer having lower salt concentration. Higher pH or higher salt is generally required for proteins. Alternatively, the elution buffer may contain an affinant designed specifically to elute the target nucleic acid or protein.

It is generally advantageous to keep the target compound in solution when not bound to the solid phase matrix. The method is therefore preferably carried out substantially in the absence of any precipitation of the target compound.

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purified target compound is provided downstream of the means for retaining the solid phase matrix.

Preferably, the first chamber communicates with further means for delivering solutions thereto.

The apparatus can be incorporated into an automatic system comprising one or more apparatus arrangements as described, together with a central control means for controlling the apparatus. The apparatus is particularly suitable for operating the method described above and can, in automated form, permit the simultaneous operation of a plurality of separate purifications in a routine manner.

Each means for delivering solutions to the respective chambers may comprise any suitable device for liquid delivery. For example, a syringe may be provided for the delivery of each buffer solution. Alternatively, a plurality of reservoirs containing the appropriate reagents may be connected by valves to each chamber, for example through a common port. In the automatic version of the apparatus the control means would be linked to the valves to ensure delivery of the correct sequence of solutions.

In a preferred embodiment, the apparatus further comprises first pressurization means for providing a positive pressure at the upstream end of the filter relative to the downstream end thereof. The purpose of the pressurization means is to force or draw liquid in the first chamber through the filter so as to separate the liquid from cells or cell debris which are retained on the filter. This may be effected by applying positive pressure upstream of the

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thereof. As discussed in relation to the first pressurization means, either positive pressure at the upstream end of the barrier or negative pressure at the downstream end of the barrier may be applied. In one embodiment, a separate waste outlet is provided in addition to the outlet for the purified target compound. Negative pressure may be applied to the waste outlet, for example using a vacuum line for the purpose of removing unwanted liquids from the solid phase matrix.

Each of the outlets may be valve controlled and in the automatic version of the apparatus, the valves would be linked to the control means.

Advantageously, the means for delivering solution and, when present, the means for delivering suspensions and the pressurization means are driven by a pneumatic fluid delivery system using continuous pressure, preferably continuous positive pressure.

Where phage-infected bacteria are used as the source material and lysed and unlysed bacterial cells are formed, a further embodiment of the apparatus may be required. In this embodiment the apparatus further comprises a pretreatment chamber upstream of the first chamber to receive lysed and unlysed bacterial cells infected with phage and a pretreatment filter, downstream of the pretreatment chamber and upstream of the first chamber, for retaining unlysed bacterial cells and bacterial cell debris.

In a preferred embodiment, a single column arrangement is used in which the filter acts to partition the first

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The present invention will now be described further, by way of example only, with reference to the accompanying drawings, in which:

FIGURE 1 shows a simple manually-operated apparatus according to the present invention for plasmid DNA;  
FIGURE 2 shows a column version of the apparatus;  
FIGURE 3 shows an automated version of the apparatus;  
FIGURE 4 shows the results of agarose gel electrophoresis comparing DNA samples prepared conventionally and samples in accordance with the present invention;  
FIGURE 5 shows a second column version of the apparatus;  
and  
FIGURE 6 shows a circuit diagram for operation of the apparatus.

In the simple embodiment of the invention shown in Figure 1, cell reservoir 1 communicates with filtrate reservoir 7 through filter 2 and three-way valve 3. Similarly, filtrate reservoir 7 communicates with outlet 11 through filter 8 and three-way valve 9. Syringes 4 and 10 are also connected respectively to three-way valves 3 and 9. Collection tube 12 communicates with outlet 11 to collect the purified sample.

In the column embodiment of the invention shown in Figure 2, the two chambers 21 and 27 are partitioned by filter 22. Further filter 28 acts as a barrier to prevent solid phase matrix in chamber 27 from passing to the remainder of the second chamber 30 and to the outlet 31. In order to deliver solutions to the chambers 21 and 27, reagents from reservoirs (not shown) enter at inlets 33 to 38. The inlets are all controlled by valves 23. Common ports 24 and 29 connect the reagent reservoirs with chambers 21 and



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bottom of the column. Pinch valve 50 is situated downstream of filter 49 to control flow from the second chamber and is monitored by means of liquid sensor 51. Indexing mechanism 54 controls the relative position of waste tube 53 or collection tube 52 according to whether liquid flowing from the column is intended for sampling or to be disposed of.

The liquid delivery system shown in Figure 6 comprises a number of reagents in reservoirs (R1 to R8) which are pressurized typically at 0.5 bar fine pressure regulated air. The exact number of reagents used will depend on the exact purification protocol to be adopted. The reagents are divided into two blocks: reservoirs R1 to R4 contain reagents to be delivered to inlet 43 of the column and are controlled by corresponding valves 61 to 64; and reservoirs R5, R6 and R8 contain reagents to be delivered to the second chamber of the column through inlet 45 under control of valves 65 and 66. Reservoir R7 contains particulate matrix material to be delivered by an independent line to the top of chamber 48 through inlet 55 under control of corresponding valves 74 and 76.

Taking delivery of reagent from reservoir R4 as an example, this reagent is delivered by opening valve 64 for a selected time period (e.g. 250ms). Because the reservoir is under pressure and there is a drop in pressure on the open side of valve 64, reagent enters the system until the valve closes. There is now a volume of liquid in the line from valve 64 to valve 68. The length and diameter of this line is chosen to accommodate the maximum likely volume. The volume of reagent is then moved to the top of the first chamber by switching valve 68 on and switching valve 67 on for sufficient time to

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suspension, valve 76 is opened to allow air to escape via the vent. Because the reservoir is sealed to form a closed system, air is drawn in via non-return valve NRV4 resulting in a bubbling action sufficient to suspend matrix particles in the reagent. Valve 76 is then closed and an appropriate dose of the reagent from reservoir 7 is removed by opening pinch valve 74. The reagent is delivered to the top of the second chamber of the column by opening valve 73 and venting via valve 71.

Where the transfer of liquid in the column from first chamber 41 to second chamber 48 is required, this is achieved by closing valves 68, 73 and 72 and allowing air to enter the top of the first chamber via valve 70. Any liquid in the first chamber is transferred to the second chamber with air being vented via valve 71. Sensor 51 provided at the column outlet assesses whether liquid has passed out of the column via valve 72. Indexing mechanism 54 is provided to move collection tube 52 into position to receive the product of the purification system.

#### Example I

In this Example the following materials were used.

Buffer (volumn/prep)	Composition
Resuspension Buffer (300 ul)	50mM Tris-HCl pH 7.5 10mM Ethylenediamine tetra acetic acid (EDTA) 100 g/ml RNase A
Lysis Buffer (300 ul)	0.2M NaOH, 1% Sodium dodecylsulphate

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solution of crude DNA was thus prepared.

Three-way valve 3 was then turned so that the entire contents of syringe 4 could communicate with filtrate reservoir 7. The contents of syringe 4 were then expelled into the filtrate reservoir 7 to which was added the matrix suspension. The crude DNA and matrix were mixed by moving syringe 10 in and out at a distance equivalent to the void volume of the matrix. The entire contents of the filtrate reservoir 7 were then filtered through filter 8 (sintered PTFE) using syringe 10 to create a vacuum. Three-way valve 9 was then switched so that the filtrate could be discarded. Wash buffer was added to the filtrate reservoir and voided as previously discussed. All residual wash buffer was removed by passing air through the filter 8 by means of plunger syringe 10. Finally, elution buffer was added to the filtrate reservoir 7, mixed by use of the syringe 10 and passed through filter 8. The purified DNA was separated from the resin in the elution buffer and collected in collection tube 12 by switching three-way valve 9.

The results of the present Example are shown in Figure 4 as a comparison with results obtained using conventional centrifugation techniques. For both methods a starter culture of 5 ml plasmid-containing E. coli K12 DH5 alpha was grown and 2 ml of the culture was used to prepare plasmid DNA. Two separate cultures were prepared by each method: one containing plasmid pBS SKII+; and one containing a clone of pBS SKII+ containing insert pFB41B2.

Table 1: Composition of reagents for DNA purification  
Examples II-IV

	Example II	III	IV
Reagents	Plasmid	M13	Blood
1	de ionised water	de ionised water	de ionised water
2	50mM Tris-HCl pH7.5 10mM EDTA 10µg/ml RNAase A	Glacial acetic acid	PBS
3	0.2M NaOH 1% SDS	4M NaClO <sub>4</sub>	20mM Tris-HCl pH8.0 5mM EDTA pH8.0
4	2.55M Potassium acetate	70% Ethanol	50mM KCl 10mM Tris-HCl pH8.3 2.5mM MgCl <sub>2</sub> 0.45% Nonidet P-40
5	200mM NaCl 20mM Tris-HCl pH7.5 5mM EDTA 70% Ethanol	10mM Tris-HCl pH7.5 1mM EDTA	200mM NaCl 20mM Tris-HCl pH7.5 5mM EDTA 70% Ethanol
6	10mM Tris-HCl pH7.5 1mM EDTA		10mM Tris-HCl pH7.5 1mM EDTA
7	Wizard DNA purification resin in 7M Guanidine HCl		Wizard DNA purification resin in 7M Guanidine HCl
Upper Filter	1 µm cellulose acetate	1 µm cellulose acetate	Leukosorb type A or B
Lower Filter	20 µm PTFE	1 µm borosilicate glass	1 µm borosilicate glass

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added to chamber 48 via tube 55, typically 500ul. The DNA binding agent is then mixed with the crude DNA by passing air into chamber 40 via line 45. The excess liquid is then removed from chamber 48 to waste. The DNA is retained on the DNA binding agent which will not pass through filter 49. A dose of reagent 5 (50% Ethanol/NaCl) is added to chamber 48 via line 45, typically 2000ul and passed through filter 49 and removed directly to waste by means of pressurising chamber 48 with air from line 44. Air is continued to be passed through filter 49 for a period that allows the filter to dry sufficiently. The indexing mechanism 54 moves the collecting tube 52 so that it is in line with the exit tube from chamber 48. The DNA is then eluted from chamber 48 by the addition of, typically, 50ul of reagent 6(TE) via lines 45 and expelled from chamber 48 by the addition of pulses of pressure via line 44.

In between liquid additions the lines are rinsed out with water (reagent 1) and via valve 68 (Figure 6).

### Example III

#### Purification protocol for M13 DNA

Different reagents are used to purify DNA from M13 but the essential elements of the apparatus are the same. The column for purifying single stranded M13 DNA has two filters: upper lum filter 46; and lower lum filter 49. The material of the filter is chosen to resist the chemicals used during the process and to have appropriate flow/binding characteristics to allow the process to occur as quickly as possible. A freshly grown culture of E.coli containing M13 to be purified is added to chamber 41, typically 1-5ml. Positive pressure is then applied to

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Positive pressure is then applied to chamber 41 by means of inlet 42. The red blood cells are then filtered through filter 46 with the excess liquid passing to waste via conduit 47, filter 49 and valve 50. The liquid waste is monitored by flow sensor 51. Once all liquid has passed the flow sensor a dose of reagent 2 (PBS) is added to the top of chamber 41 via line 43, typically 2000ul. To effect mixing chamber 48 is pressurised in short bursts via line 44 and chamber 41 is vented via inlet 42. This is done to ensure that cells caught on the filter are resuspended. A dose of reagent 3 (20TE) is added to the top of chamber 41 via line 43, typically 2000ul, to lyse any remaining red blood cells. This is then mixed as described for reagent 2. The white blood cells are then lysed with a dose of reagent 4 (lysis buffer) which is added to the top of chamber 41 via line 43, typically 500ul. This is then mixed as described for reagent 1. The resulting mixture is a cell free lysate of crude DNA. This is then transferred from chamber 41 to chamber 48 by addition of positive pressure via port 42, venting chamber 48 via line 44 and closing valve 50. A dose of reagent 7 (a DNA binding agent, e.g. silica resins, diatomaceous earth, affinity matrix) is added to chamber 48 via tube 55, typically 500ul. The DNA binding agent is then mixed with the crude DNA by passing air into chamber 48 via line 45. The excess liquid is then removed from chamber 48 to waste. The DNA is retained on the DNA binding agent which will not pass through filter 49. A dose of reagent 5 (50% Ethanol/NaCl) is added to chamber 48 via line 45, typically 2000ul and passed through filter 49 and removed directly to waste by means of pressurising chamber 48 with air from line 44. Air is continued to be passed through filter 49 for a period that allows the filter to dry sufficiently. The indexing mechanism moves the collecting

## Key to figure 6

## Symbols

## Numbers



2 way valve normally closed

61, 62, 63, 64, 65, 66, 75



2 way valve normally open

69, 73



3 way valve for liquid

68, 80



3 way valve for air

67, 70, 71, 76, 77, 78, 79



3 way pinch valve for air

74



2 way pinch valve normally open

79



Liquid column detector

LCD1



Non return valve

NRV1, NRV2, NRV3, NRV4



Reagent bottles

R1, R2, R3, R4, R5, R6, R8



Reagent bottle

R7



Flow constrictor 0.3mm i.d.

C1, C2, C3



Compressed air cylinder actuator



Air filter 0.2µm



Air filter 5µm



Pressure regulator with gauge



Variable flow control



Compressed air source



Air vent



Line to waste



Tube, inside diameter 3mm



Tube, inside diameter 0.8mm



Tube, inside diameter 0.5mm

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- (1) obtaining a cell suspension containing the target compound and optionally lysing the suspension to form a cell lysate;
  - (2) applying the suspension or lysate to a filter to remove unwanted cells and cell debris;
  - (3) contacting the filtrate with a solid phase matrix under conditions to bind the target compound to the matrix;
  - (4) separating the resultant filtrate from the matrix;
  - and
  - (5) eluting the target compound from the matrix.
7. A method according to claim 6, wherein the target compound is M13 DNA and the cells are M13 phage particles.
8. A method according to claim 6, wherein the target compound comprises a protein.
9. A method according to any one of the preceding claims, which further comprises the step of washing the matrix binding the nucleic acid or target compound to remove contaminants before eluting the nucleic acid or target compound from the matrix.
10. A method according to any one of the preceding claims, wherein the filter has a pore size in the range 0.2 to 50 microns.
11. A method according to any one of the preceding claims, wherein the step of contacting the filtered lysate with the solid phase matrix comprises adding a suspension of the matrix to the filtered lysate.



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18. Apparatus according to any one of claims 15 to 17, further comprising first pressurization means for providing a positive pressure at the upstream end of the filter relative to the downstream end thereof.

19. Apparatus according to any one of claims 15 to 18, wherein the means for retaining the solid phase matrix comprises a barrier to the solid phase matrix, which barrier is situated between at least a part of the second chamber and the outlet.

20. Apparatus according to claim 19, further comprising second pressurization means for providing a positive pressure at the upstream end of the barrier relative to the downstream end thereof.

21. Apparatus according to any one of claims 15 to 20, wherein the filter has a pore size in the range 0.2 to 50 microns.

22. Apparatus according to any one of claims 15 to 21, further comprising fluid delivery means having an outlet which is positioned to communicate with liquid when present in the second chamber.

23. Apparatus according to any one of claims 15 to 22, wherein the second chamber communicates with means for delivering thereto a suspension the solid phase matrix.

24. Apparatus according to any one of claims 15 to 23, wherein a conduit is provided to transmit the filtrate from the filter to a collection point in the second chamber.

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FIGURE 1

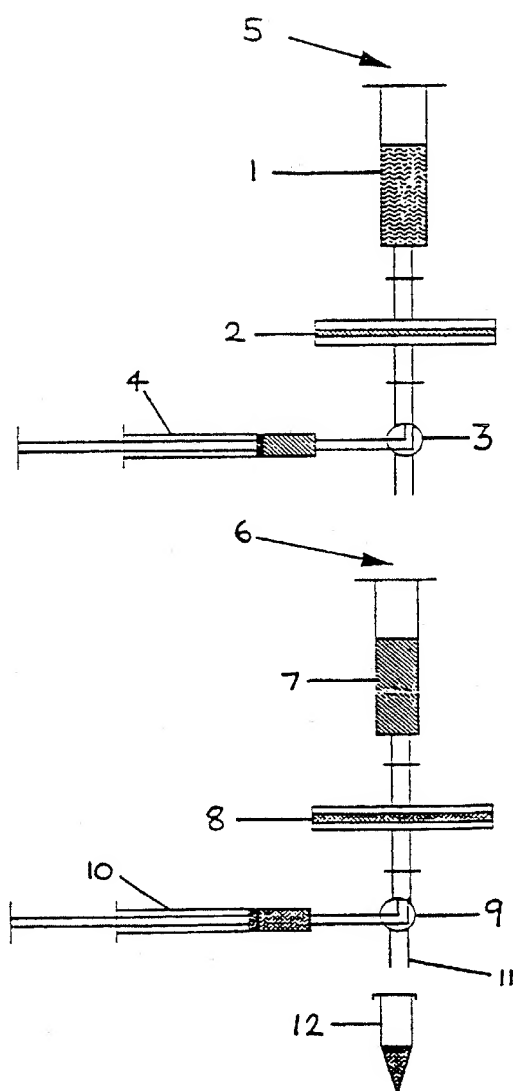
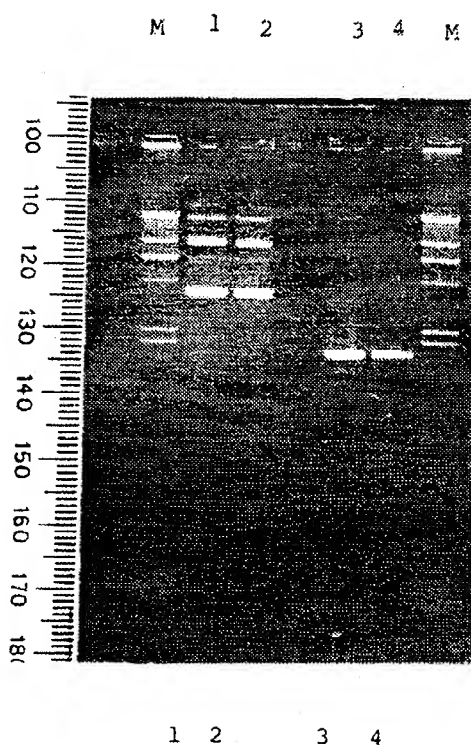


FIGURE 4



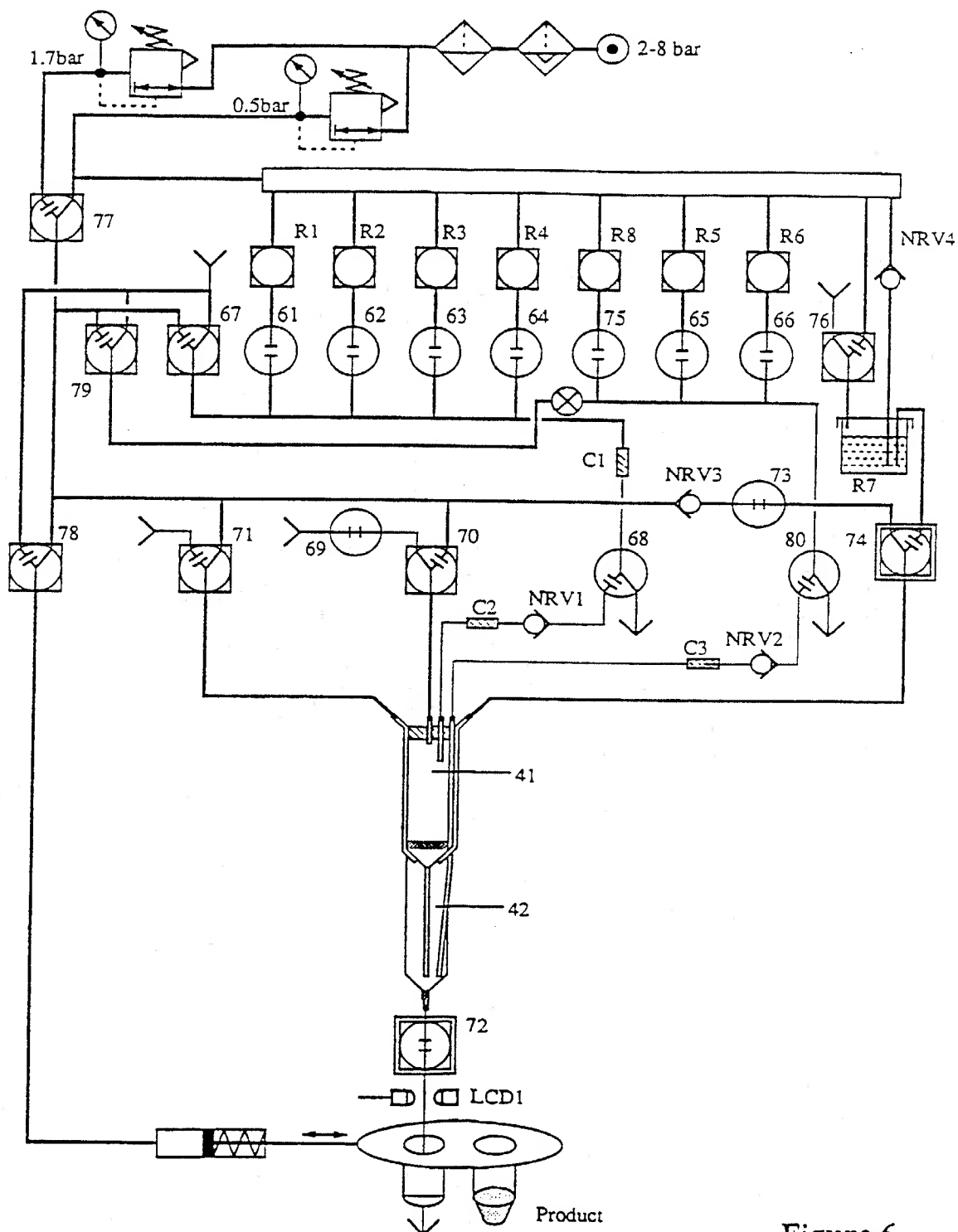


Figure 6

# INTERNATIONAL SEARCH REPORT

International Application No

PCT/GB 94/01484

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	EP,A,0 281 390 (LYLE,NELSON,REYNOLDS,WALDROP III) 7 September 1988 cited in the application the whole document ---	1-26
A	WO,A,93 11218 (DIAGEN INSTITUT FUR MOLEKULARBIOLOGISCHE DIAGNOSTIK) 10 June 1993 the whole document ---	1-26
A	US,A,4 997 932 (REARDON ET AL.) 5 March 1993 the whole document -----	1-26